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THE TURBIDITY-PRODUCING ACTION OF CLOSTRIDIUM PERFRINGENS TOXIN IN HUMAN SERA

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PREFACE

The subject of these studies was suggested to me by Professor K. O. Renkonen, M.D., Chief of the Department of Serology and Bacteriology, University of Helsinki. I am deeply indebted to Professor Renkonen for his unfailing interest and support in following and furthering the work.

I extend my sincere thanks to E. Uroma, M.D., Chief of the State Serum Institute, Professor P. Soisalo, M.D., Chief of the Kivelä Hospital, and the chiefs and physicians of several other hospitals in Helsinki, who greatly facilitated this investigation.

My thanks are also due to members of the staff of the Department of Serology and Bacteriology and of the State Serum Institute for assistance rendered to me. I especially wish to thank Miss Brita Schulman, whose help, particularly at the initial stage of the work, was most valuable.

I also thank Miss Elvi Kaukokallio for translating this report into English.

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M. Tuomioja

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INTRODUCTION

Seiffert (20) reported in 1939 the observation that a characteristic turbidity is produced when human serum and Clostridium perfringens toxin are mixed. An independent study on the same phenomenon was simultaneously published by Nagler (15), who found that this bacterium produces a turbidity in human serum used as growth medium and that the phenomenon could also be effected by the addition of Cl. perfringens toxin to human serum. He further demonstrated that the reaction could be ascribed to the lethal toxin from Cl. perfringens (Type A), that it could be inhibited by the homologous antitoxin, and that the titer of both the toxin and the antitoxin could be determined by means of this reaction.

Macfarlane *et al.* (11) found in 1941 that a similar turbidity was produced by Cl. perfringens toxin (α-toxin) in a clarified suspension of egg yolk in saline. In the same year Macfarlane and Knight (10) reported that the toxin when added to an aqueous emulsion of lecithin decomposes the lecithin and produces acid-soluble phosphorus, by measurement of which the reaction can be observed.

In 1941 Hayward (3) found that the phenomenon can be employed for the indentification of Cl. perfringens in nutrient media, and in 1943 McClean *et al.* (9) reported its use as a test for the presence of even small amounts of the toxin in infected body tissue. Renkonen (18) demonstrated in 1947 that the reaction is more rapid in hepatitis sera than in normal sera and that the results are comparable to the thymol turbidity test values.

REVIEW OF THE LITERATURE

Cl. perfringens Toxins and the Producer of the Seiffert-Nagler Reaction (SNR) [1]

The Cl. perfringens is capable of producing at least five different kinds of (exo-)toxins, *i.e.* α, β, δ, ε, and ϑ. These toxins are produced in various combinations by the different types of the bacterium. However, the α-toxin is common for all the types and it is hemolytic, necrotic and lethal. Efforts to produce the SNR with toxins other than the α have been unsuccessful. The degree of SNR produced by the different combinations of toxins is parallel with their α-toxin content as determined by other methods. It is very probable that the producer of the SNR and the α-toxin are identical (15, 11).

According to the nature of the reaction and the properties of its producer the latter has been presumed to be an enzyme (11), more specifically a phosphatase which is believed to decompose the lecithin to a diglyceride and phosphocholine (10).

SNR in Serum

When grown in human serum under anaerobic conditions the Cl. perfringens produces a turbidity in the substrate. Centrifugation will bring out three layers in the mixture: the bacteria on the bottom, an opalescent middle layer, and a creamy top layer (15). The top layer will give a »reaction of free fat» (11).

In the formation of turbidity by the toxin in human serum a distinct lag phase is first seen, after which turbidity in a more marked degree sets in (19, 2).

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[1] All the reactions referred to in the Introduction are designated in this study as the Seiffert-Nagler reaction (abbreviation SNR).

The removal of labile serum globulin does not prevent the reaction (19). Both a globulin and an albumin fraction obtained by precipitation with ammonium sulphate will produce the reaction, although it will be weaker than that obtained with the original serum (19). Inactivation of the serum has no effect on the reaction nor does storage of the serum produce any marked change in the phenomenon (20, 19). On the other hand, a weaker reaction is produced after freezing and thawing of the serum (20). Infection of the serum may make it sensitive (2). Hemolysis and turbidity will not affect the reaction (20, 2), whereas the addition of citrate (11) or antitoxin (15, 16) will intercept and prevent it. No great action is exerted by the addition of calcium ions (2) but it may promote the reaction to some extent (17).

The precipitate formed in the serum is very difficultly soluble, but it will go partially into solution in boiling alcohol or cold acetone. By means of lipid solvents the precipitate has been separated into the following components: acetone-soluble, 25 per cent; petroleum ether-soluble, 15 per cent; tetralin-soluble, 30 per cent, and protein residue, 30 per cent (2).

SNR in Egg Yolk

If an egg yolk is added to saline and the mixture passed through *e.g.* a Seitz filter a clear yellowish solution is obtained. The addition of toxin from Cl. perfringens to this solution will rapidly produce turbidity at 37° C (11). Comparison of the velocity of reaction in egg yolk and in serum will give a ratio of about 5: 1 (16).

A brief lag phase is seen also in the egg yolk reaction (11). The reaction occurs slowly at room temperature but more rapidly at 37° C. The rate also depends on the amount of toxin used. Inhibition of the reaction is effected by the addition of citrate or antitoxin (11).

In this reaction a thick layer of cream is gradually formed on the surface and the solution below becomes clarified. Fractionation of the precipitate with lipid solvents as in the case of the serum reaction gives the following values: acetone-soluble components, 40 per cent; petroleum ether-soluble, 10 per cent; tetraline-soluble, 20 per cent; protein residue, 30 per cent (2). The analysis is thus fairly analogous with that in the serum reaction.

SNR in Lecithin

If toxin from Cl. perfringens is added to a lecithin-water emulsion, acid-soluble phosphorus will be liberated (10). By estimation of this phosphorus the progress of the reaction can be closely followed.

Within certain limits, the liberated phosphorus is directly proportional to the toxin concentration. The progress of the reaction depends on the amount of lecithin used, *i.e.* when identical amounts of toxin and varying amounts of lecithin are used, the rate of reaction is at first approximately the same but slows down rapidly where the amount of lecithin is small. When approximately one-half of the substrate has been consumed, an abrupt change occurs in the rate of reaction (22).

The reaction is slow at low temperatures and gradually increases in velocity as the temperature rises. Above 25° C and up to 60° C it is approximately doubled with each increase of 10°, but thereafter it is gradually decelerated and stops completely at 69° C (22).

There is a very brief induction period in this reaction. It is more distinct in low concentrations of toxin and in the absence of calcium ions (10, 22).

Study of the effect of *e.g.* the following substances on the reaction has given the comparative figures listed below (10):

Substance	Concentration	Enzyme Activity as Compared to Activity without Added Substance
	%	%
m-Cresol	0.25	150
m-Cresol	0.025	130
Sodium Oleate	0.08	50
Sodium Taurocholate	0.08	90
Sodium Dodecyl Sulphate	0.03	5

After heating at 100° C for 10 minutes in borate buffer pH 7.6, 45 per cent of the activity of the enzyme is still present (10). The enzyme has a wide pH optimum ranging from 7.0 to 7.6 in borate buffer (10). Calcium ion accelerates the reaction, and the effect is nearly maximal in a 0.01 M $CaCl_2$ solution on toxin amounts of from 0.5 to 3.0 MLD (10). The reaction can also be followed by a manometric method if it is performed in a bicarbonate-carbondioxide buffer system (24).

Practical Applications of the SNR

Estimation of Toxin and Antitoxin Titers

The serum reaction was the first of these procedures used for estimation of the titer of the Cl. perfringens toxin. The so-called end-point method was employed. The minimum amount of toxin which produces turbidity in serum was determined and comparison was made with the minimum amount of standard toxin required for the reaction (15). The method has proved to be sufficiently accurate when comparison is made with the results of animal tests, whereas in tests for hemolysis the values have been somewhat divergent, possibly due to the dissimilar action of various amounts of calcium ions.

Estimation can be made in a similar manner of the amount of antitoxin sufficient to prevent the reaction. The antitoxin titer can be calculated by comparing this amount and the minimum amount of a known antitoxin which is capable of preventing the reaction under similar conditions (15). The combining power of the toxoid can be estimated in the same manner (16).

The egg yolk reaction can be employed similarly in all of these estimations (11). Photometric determination of the turbidity produced within a given period is also possible (5). Comparison of the turbidities with those produced by standard toxin under equal conditions will indicate the toxin titer in relation to that of the standard toxin. The titer of the antitoxin can be estimated in the same manner. The method, however, is not so well adapted for the estimation of antitoxin sera of low titers as those of high titers (17, 2).

The estimations referred to above can also be made by means of the reaction with lecithin-water emulsion (10, 24, 25). In this case the use of standard toxin or standard antitoxin will not be necessary. The method is very accurate but considerably more complicated than those described above.

Identification of Cl. perfringens

In a nutrient medium to which serum or egg yolk has been added the Cl. perfringens produces a turbidity, which on solid medium forms a circular zone around the colony (3, 4). However, several other bacteria have been found which produce a similar

turbidity, but with some exceptions the turbidity produced by the latter is not prevented by Cl. perfringens antitoxin, as is the case with that produced by Cl. perfringens. By adding this antitoxin to one side of the dish the Cl. perfringens colonies can readily be differentiated (4).

Demonstration of the Toxin in Tissue

It has been possible to demonstrate the presence of the toxin in the tissue of experimentally infected animals already within a few hours after infection (9). As the amounts of toxin produced are very small, reaction has to be made either in egg yolk or in lecithin-water emulsion.

Differences in Sera

Reaction was originally obtained in human sera alone, and even in them in 25 per cent only (20). This failure, however, can be ascribed to the method employed (19). The only human sera which do not give the reaction with the amounts of toxin usually employed were those of persons who have been given gas gangrene antiserum (15). It has later been possible to produce the reaction with most animal sera (15, 19).

In studying various human sera, differences have been found in their sensitivity toward the reaction (20, 15, 19, 2, 18). In using the end-point method it has been observed that in the toxin dilution series some sera give a weak reaction in a large number of tubes, whereas with other sera the reaction ends abruptly although it is strong in the higher toxin concentrations (2). In determining the turbidity as a function of time it has been found that the lag phase varies with different sera (2, 18).

No correlation has been observed between blood groups and the occurrence of the reaction (19). Likewise, no correlation had been seen between various diseased conditions and different modes of reaction of the sera until Renkonen (18) in 1947 demonstrated that the sera of patients with hepatitis very rapidly became turbid. He used filtrates of Cl. perfringens grown in liver broth, which produced no turbidity in normal sera within two hours at 37° C, whereas given hepatitis sera became turbid. Nephelometric determinations of the turbidity made at the end of this period

showed constantly increased values for the hepatitis sera. In comparing these nephelometric SNR values with thymol turbidity test values obtained simultaneously for the same sera he found that in most cases the positive values were strikingly coincident. The two tests were also related in respect to the strength of the reaction. In his opinion the reactions may probably be ascribed to the same mechanism, primarily the flocculation of globulins.

PLAN OF WORK

It is seen from the foregoing survey of the literature that the SNR can be produced with all human sera when an appropriate method is employed. Different sera vary in their manner of response to the Cl. perfringens toxin. Hepatitis sera give a more rapid reaction. In the latter sera the results are comparable to the thymol turbidity test values.

It was the object of the following work to observe the SNR process in serum and to study various factors which influence the reaction. Of outstanding interest in this connection was the cause of the difference between hepatitis sera and other sera. Further objects were the practical development of the method and the application of the procedure to the differentiation of pathological sera. The relationship between the thymol turbidity test and the SNR in various pathological sera also constituted one of the objects of the present study.

PERSONAL INVESTIGATIONS

Estimation of the SNR by Means of Turbidity Curves

The toxin employed was in most cases a filtrate of Cl. perfringens grown in liver broth. When the reaction was carried out in serum, I used 0.5 ml of serum which was inactivated for 30 minutes at 56° C and to which was added 0.5 ml of the toxin filtrate and 0.5 ml of physiological saline solution. In the egg yolk reaction the serum was substituted by 0.5 ml of egg yolk solution and the saline solution by 0.5 ml of borate buffer; as in the serum reaction, 0.5 ml of toxin filtrate was used. The total volume was therefore maintained at 1.5 ml, which was suitable for the nephelometer tubes employed. The reaction was carried out at 37° C. Turbidity determinations were made nephelometrically at given intervals and the increase of turbidity was stated as a function of time.

If deviations were made in any of the above factors, this is specifically mentioned in the following. A detailed descript on of the technique employed is given in the Appendix.

Turbidity Curves for the SNR in Egg Yolk

A mixture consisting of 0.5 ml each of toxin filtrate, borate buffer and egg yolk solution will produce the turbidity very rapidly — in from 5 to 15 minutes — at a temperature of 37° C even when weak toxin concentrations are used. For study of the turbidity curve as a function of time it will either be necessary to lower the temperature or dilute the toxin. The curves in Fig. 1 were obtained at a temperature of 17° C. The result was exactly the same when a weaker toxin solution and a higher temperature was used. As will be seen from the graph, there was a distinct lag phase, which was the longer the weaker the toxin, and which approximately was inversely proportional to the amount of toxin used. The lag

phase was followed by the turbidity phase, for which the curve was at first practically a straight line. The end of the curve sometimes turned downward.

As an increase in the amount of egg yolk had no effect on the initial part of the turbidity phase, the straight line might have been due to the reaction proceeding at a maximal and constant

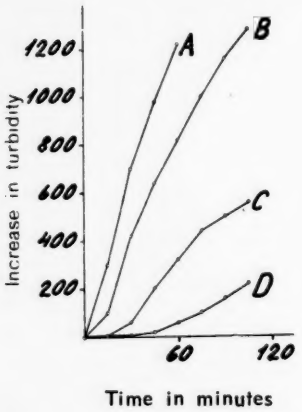

Fig. 1.

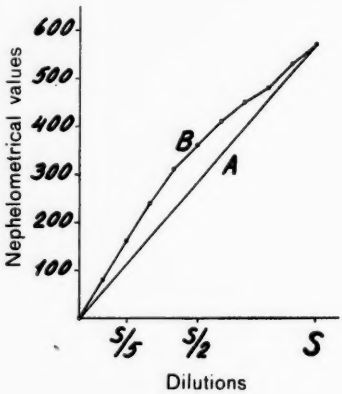

Fig. 2.

Fig. 1. — Turbidity curves for the SNR in egg yolk. Ratio of toxin in curves A, B, C, D = 8: 4: 2: 1. Temperature 17° C.

Fig. 2. — Nephelometrical values of dilutions from turbid solution (S) obtained in egg yolk reaction. A, straight line for theoretical values. B, nephelometrically observed values.

velocity. The form of the curve in the latter part of the turbidity phase was probably to some extent a result of the depletion of the substrate or of the enzyme, but for the greater part was probably due to a change in the dispersion of the turbidified substance or to the nature of the nephelometric procedure itself. Some idea of the latter is obtained from Fig. 2, which gives the turbidity values of various dilutions made from the turbid solution (S) obtained in the reaction. Should the nephelometric values correspond to the amount of substance that is rendered turbid, we would obtain values located on the straight line A; actually the nephelometric values are located on the curved line B, which slightly deviates from A.

The slope of that portion of the turbidity phase curve which forms a straight line was directly proportional to the amount of

toxin used. This slope and the length of the lag phase depended also on other test conditions, such as the temperature, the pH, and the egg yolk solution used, Although it was not difficult to maintain these other conditions constant, the properties of the egg yolk solution were liable to variation, however not greatly. Using standard toxin as a comparison the relative concentration of the toxin could nevertheless be readily determined by means

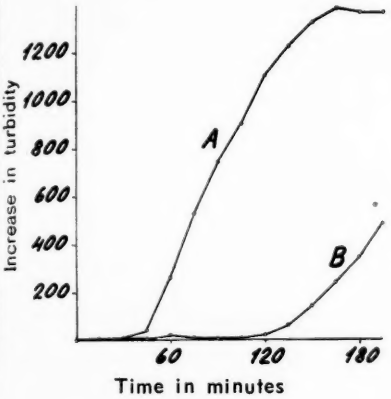

Fig. 3.

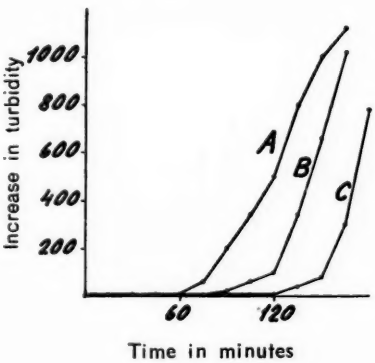

Fig. 4.

Fig. 3. — Turbidity curves for normal serum. Ratio of toxin in curves A and B = 2:1.

Fig. 4. — Turbidity curves for normal serum. Amounts of serum used: Curve A, 0.25 ml, curve B, 0.5 ml and curve C, 0.75 ml serum.

of the turbidity curves or alternatively by van Heyningen's method of determining the turbidity produced within a given time only. In the latter case the result, of course, is less reliable.

Turbidity Curves for the SNR in Normal Sera

The turb dity curve for normal serum is given in Fig. 3. Varying amounts of toxin were employed. In form the curve resembles that obtained with egg yolk, but the lag phase is much longer and the turbidity phase not so steep. Both depend upon the amount of toxin, in the same manner as in the egg yolk reaction.

The curves in Fig. 4 were obtained by varying the amount of serum used. It will be noted that with increasing amounts of serum the lag phase was slightly longer, whereas the curve for the turbidity phase was more vertical.

The form of curve reproduced in these figures is that which was usually obtained with normal serum. Both the length of the lag phase and the slope of the curve in the turbidity phase may somewhat vary even in the sera of healthy adults. Fig. 5 gives the turbidity curves for the sera of ten healthy persons and Fig. 6 shows those obtained from six different determinations carried out with one serum and gives an indication of the technical errors that

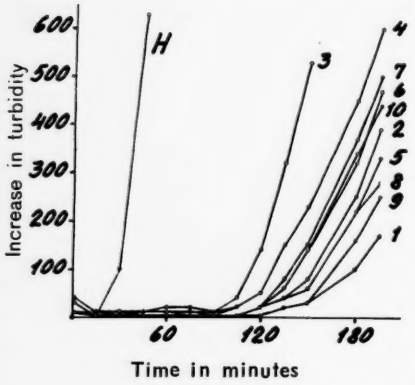

Fig. 5.

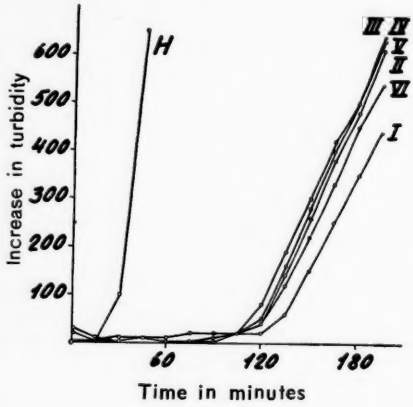

Fig. 6.

Fig. 5. — Turbidity curves for ten normal sera. Curve H, a hepatitis serum curve.
Fig. 6. — Turbidity curves for six different determinations of one normal serum. Curve H, a hepatitis serum curve.

may be present both in the performance of the test and in the reading of the results. For comparison the turbidity curve for a hepatitis serum is also shown.

The changes brought about in the curve by inactivation of the serum were slight and mostly limited to the slope of the curve for the turbidity phase.

Turbidity Curves for the SNR in Infective Hepatitis Sera as Compared with Normal Sera

The turbidity curve obtained with serum from a patient with infective hepatitis differs from the normal by its shorter lag phase. Typical turbidity curves for hepatitis serum and normal serum are shown in Fig. 7.

The length of the lag phase was liable to variation with different hepatitis sera but it was generally shorter than that of normal sera.

As sometimes was the case with normal sera, the slope of the curve for the turbidity phase of hepatitis sera was occasionally very slight, but even then it may have started to ascend at a very early stage. In the latter respect it differed from those curves for normal

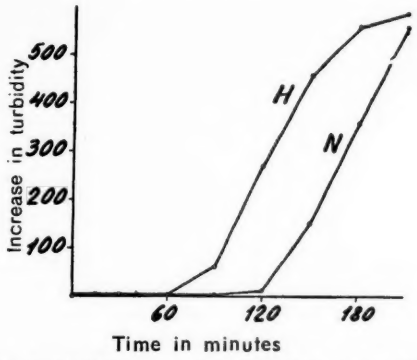

Fig. 7. — Typical turbidity curves for hepatitis serum (H) and for normal serum (N).

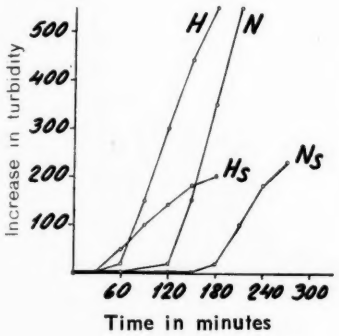

Fig. 8.

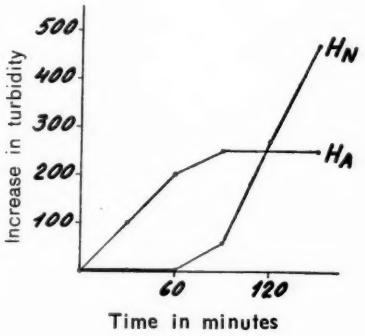

Fig. 9.

Fig. 8 — Typical (H and N) and slow (H_S and N_S) turbidity curves for hepatitis and normal sera.

Fig. 9. — Turbidity curve for a highly icteric hepatitis serum (H_A) and a typical hepatitis serum curve (H_N).

sera which had a slight slope and which regularly were seen in association with an excessively long lag phase, as in Fig. 8.

In highly icteric hepatitis sera the exceptional form of reaction shown in Fig. 9 was sometimes observed, *i.e.* the lag phase was almost absent and the curve for the turbidity phase was not very steep and reached maximum rapidly. The turbidity in these cases

differed greatly from the usual, being produced by small, very bright, oily globules which strongly refracted the light. In the case of also less icteric hepatitis sera the turbidity frequently resembled not so much a protein turbidity as in the case of normal sera.

Factors Affecting the Turbidity Curve

In connection with the SNR in lecithin-water emulsion a great deal has been written regarding the induction period produced

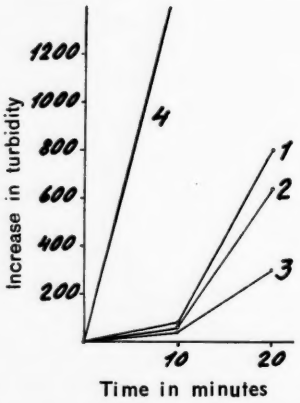

Fig. 10. — Effect of some substances on the egg yolk reaction. Curve 1, 0.03 p.c. sodium glycocholate, curve 2, 0.03 p.c. sodium taurocholate, curve 3, 1 p.c. gall bladder bile, curve 4, the control.

by the calcium ions. However, it apparently is not identical with the lag phase which occurs in the egg yolk and serum reactions but is a process of different magnitude, a very much briefer one. Both the egg yolk solution and the serum contain a fair amount of $Ca^{\cdot\cdot}$, and addition of this substance, according to Crook (2), has no marked effect upon the turbidity curve in the serum reaction.

I have studied the effect of some substances upon the latter two reactions. Fig. 10 shows fours curves obtained by adding to the egg yolk reaction (curve 4) 0.03 per cent of sodium glycocholate (1), 0.03 per cent of sodium taurocholate (2) and 1 per cent of gall bladder bile (3). Tenfold smaller amounts of sodium glycocholate and sodium taurocholate had no appreciable effect. It will be seen from the figure that simultaneously with a lengthening of the lag phase the curve for the turbidity phase became less

steep in all these three cases. The same action was apparently exerted by other factors which decelerate the velocity of reaction, such as reduction in the temperature, changes in the pH, and decrease in the egg yolk and toxin amounts.

The curve in Fig. 11 was obtained by the addition of 0.1 per cent of various fractions of serum protein to the substances in the egg yolk reaction. Fractions IV—1, IV—2 and IV—3, in particular,

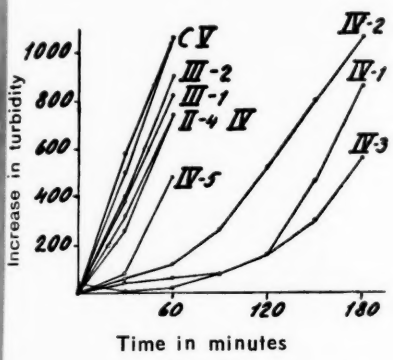

Fig. 11.

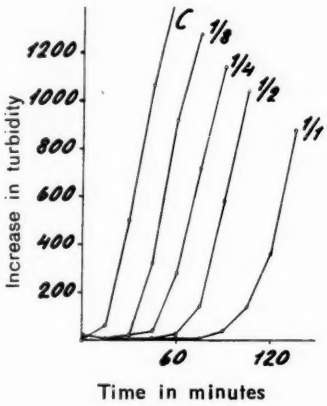

Fig. 12.

Fig. 11. — Effect of various fractions of serum proteins on the egg yolk reaction. Curve C, the control. Temperature 17° C.

Fig. 12. — Effect of varying amounts of fraction IV—1 on the egg yolk reaction. Curve C, the control. Temperature 20° C.

clearly prolonged the lag phase. As the latter two fractions were not quite clear, as explained in the Appendix, it can be said that at least fraction IV—1 prolonged the lag phase without simultaneous change in the slope of the curve for the turbidity phase. Similar effects, although weaker, were exerted by these fractions also upon the serum reaction.

The addition of varying amounts of fraction IV—1 to the egg yolk reaction gave the curves shown in Fig. 12. It will be noted that when small quantities of this fraction were added the prolongation effected in the lag phase was approximately directly proportional to the amount used.

The curves in Fig. 13 were obtained with a series of egg yolk reactions in which identical amounts of toxin but varying amounts of egg yolk solution were used, and the curves in Figs. 14 and 15

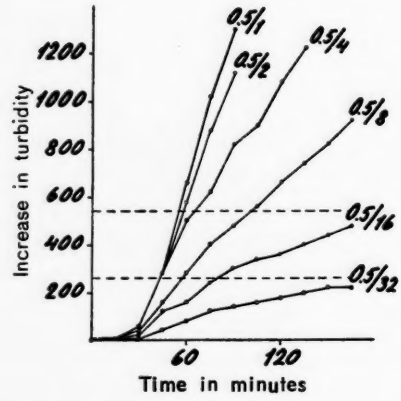

Fig. 13. — Turbidity curves obtained with varying amounts of egg yolk. Toxin constant. Temperature 20° C.

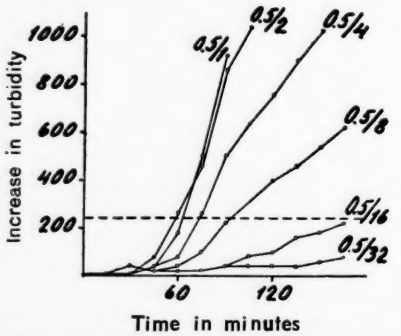

Fig. 14. — Turbidity curves obtained with a constant amount of fraction IV—1 in the egg yolk reactions. Amounts of egg yolk identical with those in Fig. 13. Toxin constant. Temperature 20° C.

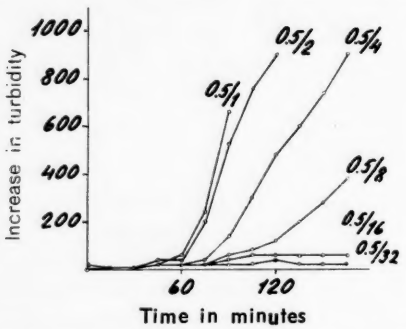

Fig. 15. — Turbidity curves for reactions made as in Fig. 14, but with twice as much fraction IV—1.

with two similar series, to each of which a different amount of fraction IV—1 was added. Each set of three curves[1] in these figures is identical except for the fact that where fraction IV—1 was added the curve begins to ascend later and the first section of the ascending part is absent. The greater the added amount of fraction IV—1 the longer the lag phase and the lower the maximum. In fact, no turbidity was produced in those cases where small amounts of egg yolk solution were employed. It does not seem

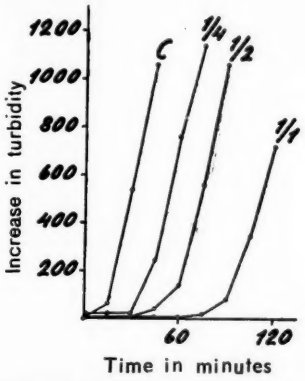

Fig. 16. — Effect of varying amounts of serum on the egg yolk reaction. Curve C, the control. Temperature 20° C.

possible to ascribe this phenomenon to a deceleration of the reaction. A simpler explanation would be that the essential reaction from its very commencement probably is similar both with and without the added fraction IV—1 and that the difference probably consists in a later onset of the precipitation — the turbidification — which occurs when the reaction has attained a given stage, dependent in this case upon the amount of fraction IV—1. Thus the action of fraction IV—1 would only consist in delay or prevention of the precipitation of the results of the reaction process.

It is probable that the essential reaction occurs also during the lag phase but is not yet manifested as turbidity.

As seen in Fig. 16, a similar prolongation of the lag phase as with added fraction IV—1 is obtained by the substitution of serum for the buffering agent in the egg yolk reaction, which will give the same reaction as when egg yolk solution is added to the serum

[1] Representing the reactions with the same amount of egg yolk.

reaction. Assuming that the substance which is acted upon by the toxin is the same in both egg yolk and serum, the addition of egg yolk will increase the amount of substrate in the serum and approximately eliminate the effect of possible variations in the amount of substrate in the different sera. This hypothesis is borne out among others by the following observation. Tests with hepatitis serum with a normal lag phase but weakly ascending turbidity curve, with normal serum with a greatly prolonged lag phase

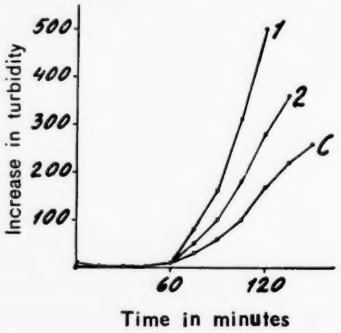

Fig. 17. — Effect of bovine albumin on the serum reaction. Curve 1, 0.1 p.c. bovine albumin, curve 2, 0.02 p.c. bovine albumin, curve C, the control.

and a particularly slow rise, and with ordinary hepatitis serum and ordinary normal serum were duplicated with addition of egg yolk. No change was seen in the correlation of the curves for the two first mentioned sera nor of those for the latter two sera; however, the curves for the slow sera moved to the position occupied by the ordinary sera. The addition of egg yolk thus altered only the correlation between the ordinary serum and slow serum curves.

In the serum reaction the turbidity phase was influenced not only by added egg yolk, which normally also shortened the lag phase, but also *e.g.* by gelatine or bovine albumin. In this case there was no change in the lag phase and only the slope of the turbidity phase curve became steeper, as seen in Fig. 17.

SNR in Mixed Sera

The SNR carried out with hepatitis serum and normal serum separately and with a mixture of these sera produced the curves shown in Fig. 18. It will be seen that the reaction in the mixed serum approached the average for the two sera.

The mixing of several normal sera gave a serum mixture in which the SNR was very similar to that obtained in mixtures which were prepared from other normal sera in a similar manner and whose curves in the coordinates were generally located in the

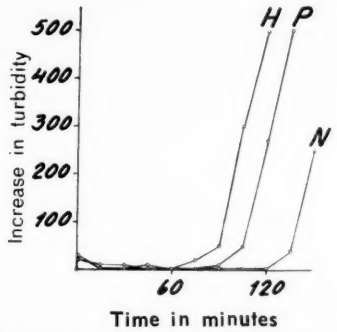

Fig. 18. — Turbidity curve for a mixture of a hepatitis and a normal serum. Curve P, 0.25 ml hepatitis serum + 0.25 ml normal serum. Curve H, 0.5 ml hepatitis serum. Curve N, 0.5 ml normal serum.

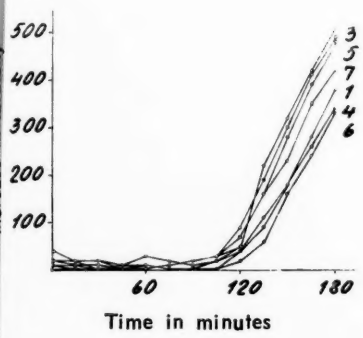

Fig. 19.

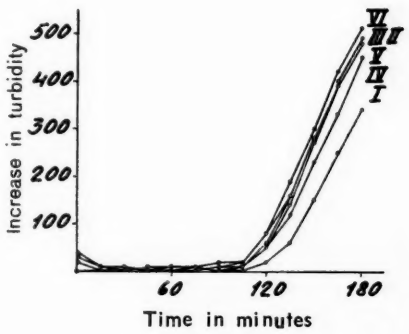

Fig. 20.

Fig. 19. — Turbidity curves for seven different serum mixtures, each comprising ten different sera.

Fig. 20. — Turbidity curves for six determinations of one serum mixture, carried out simultaneously with those in Fig. 19.

middle of the curves for the separately tested sera. A mixture of as few as ten normal sera differed from other simultaneously tested mixtures of ten normal sera by not more than the technical error in the reactions, as is seen in Figs. 19 and 20.

In view of the above described findings the serum mixture can be employed for standardization of the reaction. Fig. 21 indicates that even if there is considerable variation in the potency of the

toxin the correlation of the abscissas of any corresponding points in the turbidity curves for the standard serum mixture and for other sera remains almost constant.

Such a serum mixture may be prepared whenever the reaction is carried out with any sera. However, it is not imperative to do so, for in similarity with the individual sera the mixture is nearly stable for some length of time if stored in the refrigerator. The lag phase of the mixture, however, will gradually become very slightly prolonged and the turbidity phase less steep. A stored

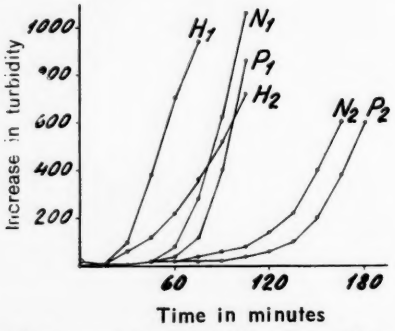

Fig. 21. — Turbidity curves for a hepatitis serum (H_1 and H_2), a normal serum (N_1 and N_2) and a standard serum mixture (P_i and P_2), using varying amounts of toxin. Ratio of toxin, curves 1/curves 2 = 2/1.

serum mixture must be sterile. The sensitization of a serum by infection, reported by Crook (2), was observed in these tests only once, in the case of a stored serum mixture which had become infected. On the other hand, no effect was observed on the manner of reaction from unsterile handling in the case of sera which had been stored for a few days in the refrigerator before testing.

A serum mixture from one to two weeks old was employed for the estimation of reaction results in examining clinical material. The numerical values for the different sera were established in the following manner. The lowest nephelometric value for each serum was designated as 0 and the abscissa of the ordinate value 200 of the standard serum curve was taken as basis of calculation. From the abscissa of this point was deducted the time which the reaction would have consumed up to ordinate value 200 had there been no lag phase. The remainder of the abscissa was divided into ten segments. The segment at which the turbidity curve of the serum under

investigation intersected this abscissa was regarded as the value of the serum under examination. Negative values might also occur. Fig. 22 illustrates this method of calculation.

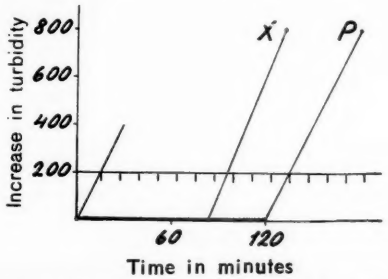

Fig. 22. — Estimation of numerical SNR values based on the turbidity curve for the standard serum mixture. Curve P, the standard serum mixture, curve X, the serum under examination, both simultaneously tested. The numerical SNR value for X = + 4.

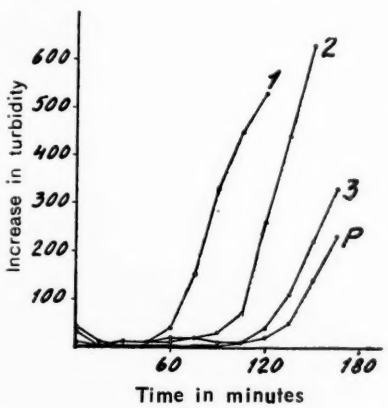

Fig. 23.

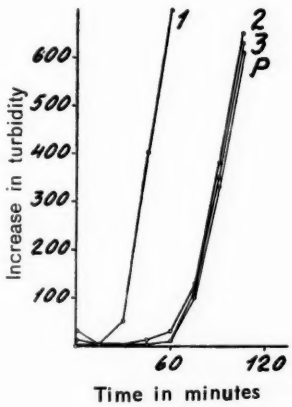

Fig. 24.

Figs. 23 and 24. — Comparison between the turbidity curves obtained with the simple serum reaction (Fig. 23) and the serum-egg yolk reaction (Fig. 24). Curve 1, a hepatitis serum, curves 2 and 3, two normal sera, curve P, a standard serum mixture.

SNR in Serum-Egg Yolk Mixture

In a later stage of this work, particularly when examining clinical material, I confined myself in an increasing degree to the serum-egg yolk reaction. I have described in the foregoing one of the advantages of this method, consisting in that those hepatitis sera which have a slow turbidity curve become similar to the ordinary hepatitis sera. The method possesses a number of other

advantages over the simple serum reaction but has the disadvantage that the form of the curve for the turbidity phase cannot be studied and observation must be limited to the relative length of the lag phase. As I have observed hardly any correlation between various diseased conditions and the turbidity phase part of the curves for their sera, although such may well exist, I have studied simple serum reaction in a limited extent only.

As a result of the addition of egg yolk solution to sera, the ascending part of the various turbidity curves becomes nearly

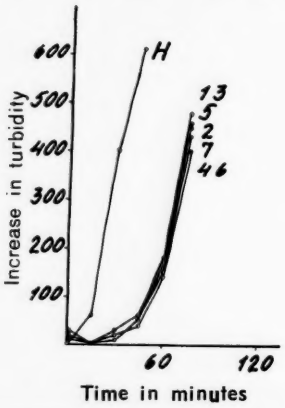

Fig. 25. — Turbidity curves illustrating technical error in the serum-egg yolk reaction. Curves 1—7 obtained with the same serum, curve H with a hepatitis serum.

uniform, and differences in the sera are seen only in the length of the lag phase. Figs. 23 and 24 show turbidity curves obtained with the same sera when employing the serum and serum-egg yolk methods.

The curves in Fig. 25 were obtained with one serum by carrying out the serum-egg yolk reaction in several tubes.

Fig. 26 presents four curves showing the effect of different degrees of hemolysis upon the reaction. Curve 1 covers serum hemolyzed to 1/100 of the total hemolysis of the blood, curve 2 to 1/500, curve 3 to 1/1,000, and curve 4 no hemolysis.

The three curves in Fig. 27 were obtained by adding small amounts of turbid serum to clear serum from the same person before carrying out the reaction, the initial nephelometric value being 588 in curve 1, 435 in curve 2, and the initial value of the original clear serum 154.

These tests indicate that both a strong hemolysis and a heavy initial turbidity influence the curves. In hemolysis curve 1, however, the initial nephelometric value was already high, and the

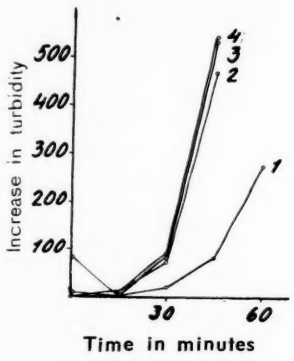

Fig. 26.

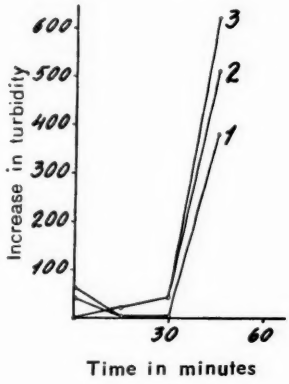

Fig. 27.

Fig. 26. — Effect of hemolysis of the blood on the serum-egg yolk reaction. (See p. 28)

Fig. 27. — Effect of the original turbidity of serum on the serum-egg yolk reaction. (See p. 28)

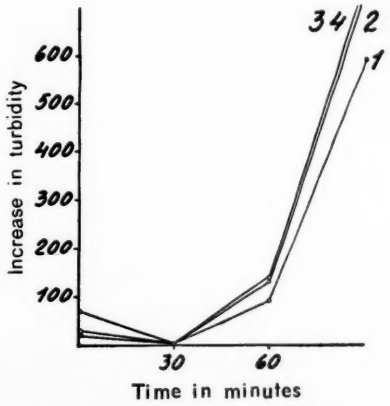

Fig. 28. — Effect of addition of bilirubin on the serum-egg yolk reaction. (See p. 30)

deceleration of the turbidity rate was probably due also to the initial turbidity. However, the effect of the turbidity may to a great extent be a result of the nephelometric estimation. It is well to avoid great differences in the original degree of turbidity of the sera when it is desired to compare the results.

The four curves in Fig. 28 indicate the effect of added bilirubin on the turbidity curve. The addition was 4 mg per 100 ml in the case represented by curve 1, 2 mg per 100 ml in curve 2, and 1 mg per 100 ml in curve 3. Curve 4 shows the control. It will be noted that these amounts of bilirubin had no appreciable effect on the reaction.

Clinical Investigations

Examination of all the clinical cases was made by the serum-egg yolk reaction, supplemented in some cases by the serum reaction. The thymol turbidity test (TTT) was also carried out with all the sera. The sera had been submitted to the laboratory for the WR test and at the time the SNR was performed one to three days had passed since the drawing of the sample. Immediately prior to examination the sera were inactivated at 56° C for 30 minutes.

Control Sera

As control sera were employed sera submitted by the blood center and some obtained in mass examination of students. Strongly hemolytic or turbid sera were discarded.

A total of 211 these normal sera were examined and their distribution according to SNR values is shown in Table 1. Many normal sera

TABLE 1

NORMAL SERA

SNR Value	No. of Cases
+4	6
+3	46
+2	77
+1	71
−1	11
Total	211

which gave a value of +4 did not distinctly differ in respect to the lag phase from sera with lower values but the rise in the turbidity curve was more than usually steep already before the ordinate value of 200.

Sera from ten aged healthy persons (average age 72 years) were also examined and the SNR values are shown in Table 2.

TABLE 2

SERA OF AGED PERSONS

SNR Value	No. of Cases
+3	1
+2	4
+1	5
Total	10

Sera from Clinical Cases

The sera were chosen at random but care was taken that all hospital departments contributed. However, a larger number of icteric sera were included, whereas strongly hemolytic and very turbid sera were rejected.

There were 357 sera from clinical cases. Their distribution according to SNR values and hospital departments is seen in Table 3.

TABLE 3

DISTRIBUTION OF SNR VALUES ACCORDING TO HOSPITAL DEPARTMENTS

SNR Value	Dermato-logical Dept.	Pediatric Dept.	Gynecological & Obstetrical Dept.	Ophthalmo-logical Dept.	Otological Dept.	Nervous & Mental Disease Dept.	Surgical Dept.	Medical & Epidemical Dept.	Radiological Dept.	Total
+10	—	—	—	—	—	—	—	—	—	—
+ 9	—	—	—	—	—	—	—	1	—	1
+ 8	—	—	—	—	—	—	1	3	—	4
+ 7	—	1	—	—	—	—	1	6	—	8
+ 6	—	—	—	—	—	—	—	15	—	15
+ 5	—	2	—	—	—	—	—	12	—	14
+ 4	—	2	1	1	—	—	5	13	3	25
+ 3	—	—	2	—	—	4	11	27	11	55
+ 2	2	1	3	5	3	11	5	49	14	93
+ 1	6	1	4	2	2	5	6	39	18	83
+ 1	2	—	2	—	—	2	5	14	7	32
− 2	—	—	2	—	—	1	5	3	8	19
− 3	—	—	2	—	—	—	1	—	4	7
− 4	—	—	—	—	—	—	—	—	—	—
− 5	—	—	—	—	—	—	—	—	—	—
− 6	—	—	—	—	—	—	—	1	—	1
Total	10	7	16	8	5	23	40	183	65	357

The sera of the patients listed in Table 4 (diagnosis by the hospital) differed from the normal serum SNR values of +4 to −1.

TABLE 4

DIAGNOSIS, SNR AND TTT VALUES, AND HOSPITAL DEPARTMENT [1] OF CASES WITH SNR VALUES DEVIATING FROM THE NORMAL

SNR Value	TTT Value	Diagnosis	Hospital Department
+9	15	Hepatitis ac.	M & E
+8	2	Ca pancreatis (Necrosis cutis nuchae)	S
+8	8	Hepatitis epid.	M & E
+8	1	Sepsis (p. abort. inf. Bronchopn. l.s.) (Hepatitis ac.)	M & E
+8	13	Hepatitis ac.	M & E
+7	0	New-born (Umbilical cord serum)	P
+7	22	Hepatitis ac.	S
+7	19	Hepatitis epid.	M & E
+7	10	Hepatitis epid.	M & E
+7	14	Hepatitis ac.	M & E
+7	—	Hepatitis ac.	M & E
+7	12	Hepatitis ac. Gravid. m.V	M & E
+7	1	Ca pancreatis (ventriculi?) c.icter. et metast. hepatis? Hypert. art. Myodeg. cordis	M & E
+6	15	Vitium cong.? cordis. Endoc. lenta. Convalesc. p. pneum. l. dx. Alc. chr. Anaemia sec.	M & E
+6	12	Hepatitis epid. Hepatargia	M & E
+6	2	Hepatitis epid. (Lues seroneg. med.)	M & E
+6	14	Hepatitis epid.	M & E
+6	5	Hepatitis ac.	M & E
+6	9	Hepatitis ac.	M & E
+6	7	Hepatitis ac. Sequele p. infarct. cordis. Tub. pulm. l. dx. (inveter.)	M & E
+6	1	Hepatitis ac. Infiltr. pulm. l. dx. Pneumon. atypica?	M & E
+6	24	Hepatitis ac. epid.	M & E
+6	15	Hepatitis epid.	M & E
+6	—	Hepatitis epid. Anaemia normochr.	M & E
+6	—	Hepatitis ac. Gastroduodenitis	M & E
+6	0	Stenosis ostii atr. ventric. sin. Ins. valv. mitr. Stenosis ostii aortae. Myod. et insuff. cordis. Hydrothorax l. a.	M & E

[1] M & E = Medical & Epidemical Dept., S = Surgical Dept., P = Pediatric Dept., G & O = Gynecological & Obstetrical Dept., R = Radiological Dept., N & M = Nervous & Mental Disease Dept.

TABLE 4 (Continued)

SNR Value	TTT Value	Diagnosis	Hospital Department
+6	0	Intoxicatio c. eau de col.	M & E
+6	0	Intoxicatio c. eau de cologne. Emphys. pulm. Alc. chr.	M & E
+5	0	New-born (Umbilical cord serum)	P
+5	4	Septicaemia. Gastroenteritis ac. Bronchopneumonia. Leptomening.	P
+5	1	Anaemia hyperchr. gravis. Cholecystopathia	M & E
+5	1	Polyarthritis chr. Myxodema. Blepharitis squamosa o. a.	M & E
+5	2	Hepatitis epid.	M & E
+5	6	Hepatitis epid. Parotitis epid.	M & E
+5	8	Hepatitis ac.	M & E
+5	6	Hepatitis ac.	M & E
+5	2	Hepatitis ac. Gravid. m. V	M & E
+5	0	Intoxicatio c. eau de cologne. Alc. chr.	M & E
+5	0	Pneumonia l. a. intest.	M & E
+5	14	Hepatitis epid.	M & E
+5	4	Hepatitis ac.	M & E
+5	0	Leuc. lymphatica. Herpes zoster necr. Anaemia sec.	M & E
−2	2	Residua p. abort. m. II—III	G & O
−2	0	Partus. Endometritis levis	G & O
−2	0	Alcohol. chr.	N & M
−2	7	Cholecystopathia. Lues seropositiva med.	S
−2	1	Append. ac. gangr.	S
−2	2	Cholecystopathia chr.	S
−2	0	Haemangioma cavern. fem. sin.	S
−2	0	Hypernephroma? Tumor renis dx.	M & E
−2	0	Tub. pulm. exs. cav. gr. III l. a. Residua p. pneumoth. art. Tub. laryng.	M & E
−2	0	Tumor pulm. sin. Tumor ovarii l. dx. Myomata uteri. Pneumonia l. sin.	M & E
−2	0	Ca (?) pulm. dx. inop.	S
−2	1	Lues	R
−2	1	Mastitis chr. cystica	R
−2	0	Hypernephroma renis dx.	R
−2	0	Ca pulm. sin. Metast. pariet. abd. Tub. pulm.	R

TABLE 4 (Continued)

SNR Value	TTT Value	Diagnosis	Hospital Department
−2	2	Tumor pulm. l. dx.	R
−2	0	Ca colon. transv.	R
−2	0	Lymphogranulomatosis	R
−2	0	Ca hypopharyngis. Metast. col. vert.	R
−3	1	Partus. Endometritis levis	G & O
−3	2	Partus. Thrombocytop. Polyarthritis. Asthma bronch.	G & O
−3	0	Hypernephroma?	S
−3	3	Tumor renis dx. inop.	R
−3	0	Pneumonia chr. dx.	R
−3	0	Ca pulm. dx.	R
−3	0	Sarcoma femoris sin.	R
−6	1	Ca gl. parotis c. met. hep.	M & E

Liver Diseases

There were 57 cases of liver diseases, 36 being cases of hepatitis and 21 of other pathologic conditions. The diagnoses and the SNR and TTT values are shown in Table 5.

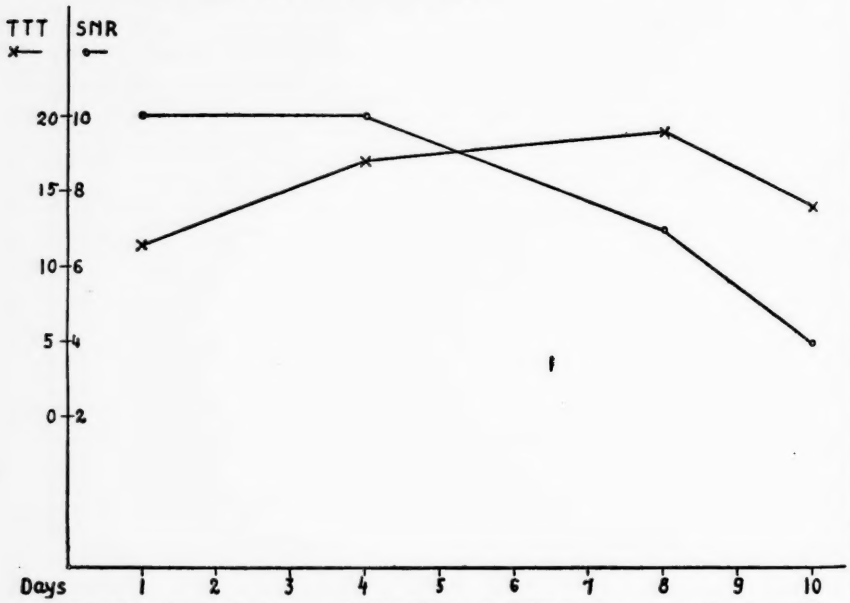

Fig. 29. — Typical changes in the TTT and SNR in the serum of a hepatitis patient during course of illness.

TABLE 5

DIAGNOSIS AND SNR AND TTT VALUES OF CASES WITH LIVER DISEASES

No.	Hepatitis	SNR Value	TTT Value	No.	Other Liver Diseases	SNR Value	TTT Value
1	Hepatitis ac.	+9	15				
2	Hepatitis epid.	+8	24				
3	Hepatitis ac.	+8	13				
4	Hepatitis epid.	+7	19	1	Ca pancreatis cum		
5	Hepatitis epid.	+7	10		ictero et metast.		
6	Hepatitis ac.	+7	14		hepatis. Hypert.		
7	Hepatitis ac.	+7	—		art. Myodeg. cordis	+7	1
8	Hepatitis ac. Gravid. m. V	+7	12				
9	Hepatitis ac.	+7	22				
10	Hepatitis epid. Hepatargia	+6	12				
11	Hepatitis epid. (Lues seroneg. med.)	+6	2				
12	Hepatitis epid.	+6	14				
13	Hepatitis ac.	+6	5				
14	Hepatitis ac.	+6	9				
15	Hepatitis ac. Sequele p.inf. cordis. Tub. pulm. l.dx. inveter.	+6	7				
16	Hepatitis ac. Infiltr. pulm. l.dx. Pneum. atypica	+6	1				
17	Hepatitis ac. epid.	+6	24				
18	Hepatitis epid.	+6	15				
19	Hepatitis epid. Anaemia normochr.	+6	—				
20	Hepatitis ac. Gastro-duodenitis	+6	—				
21	Hepatitis epid.	+5	2	2	Anaemia hyperchr.		
22	Hepatitis epid. Parotitis epid.	+5	6		gravis.Cholecystop.	+5	1
23	Hepatitis ac.	+5	8				
24	Hepatitis ac.	+5	6				
25	Hepatitis ac. Gravid. m. V	+5	2				
26	Hepatitis epid.	+5	14				
27	Hepatitis ac.	+5	4				

TABLE 5 (Continued)

No.	Hepatitis	SNR Value	TTT Value	No.	Other Liver Diseases	SNR Value	TTT Value
28	Hepatitis epid.	+4	3	3	Thrombocytopenia sympt.		
29	Hepatitis epid.	+4	16		Sugill.reg.var.corp.		
30	Hepatitis ac.	+4	3		Epistaxis. Anaemia posthaem. gravis. Myod. et ins.cordis.		
					Ca hepatis??	+4	1
				4	Arterioscl. Myod. et ins. cordis. Cholelithiasis	+4	0
				5	Abortus m. II inf. Uraemia et Icterus. Complexus hepat. Pyelonephr. colica. Anaemia sec.	+4	1
31	Hepatitis ac. Polyarthritis levis	+3	0	6	Cirrhosis hepatis? Myod. et ins.cordis.		
32	Hepatitis ac.	+3	0		Alcoh. chr.	+3	11
33	Hepatitis. Graviditas m. VII	+3	4	7	Cholecystitis. Cholelithiasis	+3	0
				8	Cholecystopathia	+3	0
				9	Appendicitis ac? Cholecystopathia?	+3	0
34	Hepatitis epid.	+2	0	10	Cholecystopathia	+2	0
				11	Cholecystitis	+2	1
35	Hepatitis ac.	+1	12	12	Hypert. art. Cholecystopathia	+1	0
36	Hepatitis ac.	+1	1	13	Icterus obturationis (Cholelithiasis?)	+1	1
				14	Cholelithiasis	+1	0
				15	Cholelithiasis	+1	0
				16	Choledocholithiasis	−1	0
				17	Ca hepatis?	−1	0
				18	Cholecystitis ac. perforans. Periton.diff. incip. Acc. Pancreatitis	−1	2
				19	Cholecystopathia chr.	−2	2
				20	Cholecystopathia	−2	7
				21	Ca gl. parot. cum metast. hepatis	−6	1

Apart from the above mentioned series of cases the changes occurring in the SNR and TTT of ten patients with infective hepatitis were observed as the disease progressed. In these cases the reactions were carried out three or more times in the course of the illness. The highest SNR values were obtained at the first examination in all the cases, with one patient as early as two days after the appearance of the icteric color. The SNR value generally declined quite rapidly, usually within two or three weeks. In most cases the TTT values, on the other hand, did not reach maximum at the first examination but an increase occurred in the early stages of the disease. The TTT values declined gradually later but much more slowly than the SNR values. Two cases were normal throughout in respect to the TTT although the SNR was highly positive. At least one of these cases was probably an inoculation hepatitis. Fig. 29 illustrates a typical movement of the values in the course of the disease.

Pregnancy

The clinical series included 14 cases of abortion, pregnancy and parturition, for which the SNR and TTT are given in Table 6.

TABLE 6

CASES OF PREGNANCY, DELIVERY AND ABORTION

No.	Diagnosis	SNR Value	TTT Value
1	Sepsis (p.abort.inf.) (Bronchopn. l.s.) (Hepatitis ac.)	+8	1
2	Hepatitis ac. Graviditas m. V	+7	12
3	Hepatitis ac. Graviditas m. V	+5	2
4	Abort. m. II inf.	+4	1
5	Hepatitis ac. Grav. m. VII	+3	4
6	Partus. Hypertonia ess. Albuminuria	+3	0
7	Graviditas m. III. Hyperemesis	+2	2
8	Partus	+1	1
9	Graviditas m. V. Lues seroneg.	+1	1
10	Partus	−1	0
11	Partus	−2	0
12	Residua p. abort. m. II—III	−2	2
13	Partus	−3	2
14	Partus	−3	1

The cases of parturition, in which the samples of blood were drawn from one to two days after delivery, generally gave negative SNR values. As there was no indication in these instances as to whether this was the case also in pregnancy, eight cases of late pregnancy were examined outside the original study material. These cases are given in Table 7 and show, with one exception, negative SNR values.

TABLE 7

PREGNANCY SERA

No.	Month of Pregnancy	SNR Value
1	9	−5
2	8	−5
3	9	−3
4	8	−3
5	7	−2
6	8	−2
7	9	−2
8	6	+1

New-Born

The clinical series included two cases of umbilical cord sera. Both gave high SNR values, as seen from Table 4. Outside the original series, twenty-three umbilical cord sera were examined. These cases are given in Table 8. They show high values for SNR. TTT values were completely negative.

TABLE 8

UMBILICAL CORD SERA

SNR Value	No. of Cases
+8	1
+7	5
+6	4
+5	10
+4	3
Total	23

Intoxication by Hair Tonic

There were nine cases of »intoxication with eau de Cologne» resulting from the drinking of the same hair tonic, which probably contained carbon tetrachloride (1). The SNR values for these cases are shown in Table 9. The TTT later became weakly positive in some of the cases.

TABLE 9

CASES OF INTOXICATION BY HAIR TONIC

No.	SNR Value	TTT Value
1	+6	0
2	+6	0
3	+5	0
4	+4	1
5	+4	1
6	+3	0
7	+3	1
8	+2	0
9	+2	0

Tumors

The series included 92 cases of tumor. In numerous cases the growth was located in the female reproductory organs (30 cases) or in the mammary glands (7 cases). Normal values were obtained in these cases. Renal tumors were present in five cases. Four of these, which gave negative values deviating from the normal, are listed in Table 4. In the fifth case the diagnosis was tumor renis sin., aplasia renis dx., and the values were −1 for the SNR and 1 for the TTT. There were pulmonary tumors in twelve cases; six of these cases, in which the values −2 and −3 were obtained, are listed in Table 4. The values for the other patients were +1 in three cases, +2 in two cases, and +3 in one case. Tumors of the pancreas were suspected in three cases, two of which yielded the values of +8 and +7 and are included in Table 4. The diagnosis in the third case was anaemia hypochrom. (tumor pancreatis?), myod. et insuff. cordis, helminthiasis, and the SNR value was +4. There were myelomas in three cases, which gave SNR values of −1, −1 and +3 and TTT values of 32, 0 and 10. In five other

cases of tumor, values deviating from the normal were obtained and are listed in Table 4. It deserves to be noted that the cases of tumor which gave negative values did not include clinically malignant tumors alone but also other types, mostly of a large size.

Other Diseases

The study series contains certain isolated cases or small groups of cases which may warrant specific mention, such as two cases of lymphatic leucemia in which the SNR values were +5 and +4 and the TTT values 0 and 0, one case of acute myeloid leucemia with an SNR value of +2 and a TTT value of 1, one case of chronic monocytic leucemia with SNR +2 and TTT 2, and two of epilepsia with SNR values of −1 and −1, and TTT values of 0 and 0.

Cases of renal lesion gave values which were within normal range but nevertheless very low.

In collaboration with other workers I have earlier examined ten cases of acrodermatitis chronica atrophicans Herxheimeri, in nine of which the TTT values were high although the SNR values were normal (8).

Simple Serum Reaction

The simple serum reaction was carried out in about one-half of the clinical cases. The results given by the hepatitis sera in this test were very similar to those obtained in the serum-egg yolk reaction. However, numerical expression of the results is considerably more inaccurate, for reasons already explained.

Correlation between various diseased conditions and the slope of the curve for the turbidity phase in the serum reaction was seen only in diabetes, in which the curve was constantly very steep.

Color of the Sera

Estimation of the color of the sera was made by the naked eye. Most of the sera which gave positive values were of course yellow, but some exceptions were seen. Some yellow sera gave negative values (*e.g.* in gall bladder disease). In a large number of those cases of tumor, pregnancy and parturition in which low values were obtained the serum was of a very light color. There were, however, exceptions in all directions.

DISCUSSION

It is my opinion that the SNR in serum or egg yolk solution is to be so comprehended that the action of the toxin commences immediately but its effect is manifested as a turbidity only after a certain degree of reaction has been reached. This degree is dependent on the capacity of the serum to prevent turbidification. It is possible that this power to prevent the occurrence of turbidity is a significant factor also in other colloidal reactions in serum, although it doubtlessly varies according to the substance which is being rendered turbid as well as according to other test factors. With hepatitis sera this capacity must probably be regarded as lowered. It is not definitely clear whether fraction IV—1 is in any way connected with this capacity. Such a supposition is contradicted by the fact that fraction IV in itself, in which also fraction IV—1 is contained, does not lengthen the lag phase. Volkin *et al.* (23), on the other hand, in studying non-specific reactions in syphilis, observed that fraction IV—1 greatly prevents the occurrence of these reactions. They were unable to isolate this factor from icteric sera. Similarly, Martin (14) has demonstrated that the α-globulin fraction has an inhibitory action on the thymol turbidity test. The probable action of α-globulin in the SNR is to some extent borne out by the negative values obtained by me with hypernephroma sera which, according to the literature, have a very high α-globulin content. It is possible, however, that a state of balance to which a number of factors contribute is here present.

If we assume that turbidification in the TTT is inhibited by the same factor which in the SNR prolongs the lag phase and which we here may designate as A, and if we further assume that the TTT is influenced also by the amount of substance which is rendered turbid, here called B, we may interpret the diverging results of the SNR and TTT so that in order to give a positive TTT the

ratio A: B must be smaller than normally. The following alternatives may then be obtained:

A: B	A	B	SNR	TTT	Example of Sera
Normal	Normal	Normal	Normal	Normal	Normal serum
Reduced	Normal	Increased	Normal	Positive	Acrodermatitis [1] / Late hepatitis
Reduced	Reduced	Normal	Positive	Positive	Incipient hepatitis
Reduced	Reduced	Increased	Positive	Positive	Hepatitis
Increased	Normal	Reduced	Normal	Normal	
Increased	Increased	Normal	Negative	Normal	Pregnancy / Hypernephroma
Increased	Increased	Reduced	Negative	Normal	
?	Reduced	Reduced	?	?	Umbilical cord serum
?	Increased	Increased	?	?	

[1] Very high γ-globulin values are seen in acrodermatitis (7).

The above is merely an attempt to solve in theory the diverging results obtained in the different colloidal reactions. However, when the question is of colloidal reactions, the old concept of serum lability should in my opinion be ascribed to variations both in the amount of the turbidified substance and in the capacity of the serum to inhibit turbidification.

Another point to which I wish to draw attention is the importance of the serum mixture in the practical application of the SNR. It should be possible to measure the titer of the Clostridium perfringens toxin without the use of a known standard toxin by employing a mixture of normal sera provided the experimental conditions and the method of estimation were agreed upon.

When the measurement of weak antiserum is made by the serum or egg yolk reaction, the non-specific inhibition produced by the antiserum should be taken into consideration.

CONCLUSIONS

Following a lag phase of varying length, toxin from the Cl. perfringens produced a turbidity in serum. The length of the lag phase was inversely proportional to the amount of toxin, and the velocity of the turbidification which thereupon followed was directly proportional to it. The same was true of the reaction in egg yolk.

The lag phase and the turbidity phase varied in different sera. In hepatitis serum the lag phase was shorter than in normal serum.

The addition of egg yolk solution to the serum reaction rendered the reaction of different normal sera similar. Sera from certain clinical cases which were studied differed partly from normal sera in respect to the lag phase, *viz.*:

Lag Phase	*Sera*
Short	Umbilical cord sera
»	Hepatitis sera
Long	Late pregnancy sera
»	Certain tumor sera

Comparison of the SNR and TTT values obtained in the present study gives the following list of those values which diverge from he normal.

SNR	*TTT*	*Sera*
Positive	Normal	Umbilical cord sera
Negative	»	Late pregnancy, and certain tumor sera
Normal	Positive	Acrodermatitis sera
Positive	»	Hepatitis sera

A fifth group might comprise sera which gave a negative SNR but a positive TTT. However, only one serum of this kind was seen in these tests, in a case of cholecystopathia.

Hepatitis sera gave positive results both in the SNR and the TTT. In early hepatitis the SNR was sensitive, but as the disease progressed this reaction became normal more rapidly than the TTT. Positive results were obtained in both reactions with also other than hepatitis sera.

APPENDIX

SNR Technique

Toxin

The toxin used in this study was a filtrate of Cl. perfringens grown in liver broth. The substrate was prepared as follows: 1 liter of meat water, 5 grams of sodium chloride and 10 grams of peptone (Difco Bactopeptone) were boiled on two consecutive days and passed through a filter. A large quantity of liver hash was added, the pH was adjusted to 8.0 and the mixture autoclaved.

In preparing the toxin a number of strains of Cl. perfringens were used. No difference was seen in the action of the toxins obtained.

Parallel with the above described filtrate I employed in a part of the tests dry toxin either prepared by myself by precipitation with $(NH_4)_2SO_4$ or furnished through the kindness of the Danish State Serum Institute. The test dosage of the latter was stated to be 0.63 mg per 0.1 AE in intracutaneous tests on guinea pigs, and 0.31 mg of this toxin in 1 ml of buffer solution was equal to the strength generally used by me in the SNR. This potency produced turbidity in egg yolk solution in 10 minutes and in a mixture of egg yolk and serum in from 1 to 1½ hours.

Sera

The sera had been submitted for WR, and at the time of my carrying out the SNR from 1 to 3 days had passed from their withdrawal. The sera were inactivated at 56° C for 30 minutes prior to testing.

Egg Yolk Solution

To prepare the egg yolk solution one egg yolk was mixed in 250 ml of physiological saline solution, the mixture was shaken, adjusted to pH 7.4 and passed through a Seitz filter.

Buffer Solution

For tests in which no serum was used I employed a buffering agent prepared from two solutions as follows:

Solution I: 19.108 g of $Na_2B_4O_7 \cdot 10\,H_2O$ per liter of H_2O,
Solution II: 12.404 g of $H_3BO_3 + 2.925$ g of NaCl per liter of H_2O.

For the buffer solution 100 ml of solution I and 900 ml of solution II were mixed, which gave a pH of 7.36.

Serum Protein Fractions

The protein fractions were received from E. Cohn, Harvard University. Solution was made in water or saline. Considerable difficulty was encountered in dissolving several of the fractions, probably due in part to their age. The lipid fractions in particular remained partly undissolved; the undissolved residue was removed by filtration. Some of the fractions remained opalescent. This was true especially of fractions IV—2 and IV—3, and the results obtained in the SNR in these cases are probably to be ascribed to the turbidity of the fraction itself. Fraction IV—1, on the other hand, formed a solution that was entirely clear.

Nephelometer

Use was made of a Zeiss nephelometer with wedge light, red light, comparison window No. 4 and red occular filter L1/39.

Performance of the SNR

I measured into specially selected narrow tubes 0.5 ml of serum and 0.5 ml of egg yolk solution or physiological saline solution, after which 0.5 ml of toxin filtrate was added to each tube. The tubes were shaken and the initial turbidities were measured. Each tube was immediately placed in a water bath at 37° C. The tubes were removed from the bath in the same order for re-estimation and thereafter replaced.

The test results for each tube were plotted, using the testing time as the abscissa and the progress of turbidity as the ordinate. The curve was compared with a standard curve obtained simultaneously with a mixture of ten normal sera.

Thymol Turbidity Test Technique

The TTT was carried out in the standard manner (12, 13, 21, 6). All the sera were 1 to 3 days old and inactivated, except the sera from cases of acrodermatitis and those from such cases of hepatitis as were examined several times during the illness; these were all freshly tested. Thirty minutes after the addition of the reagent the turbidity produced in the reaction was estimated by means of a Zeiss photometer with filter S66/38. The values obtained were converted to Maclagan units by comparison of the turbidity with that of $BaSO_4$ (21, 6).

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